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Gene Transfer Using Replication-Defective Human Foamy Virus Vectors

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Replication-defective vectors based on an infectious molecular clone of human foamy virus (HFV) were constructed by deletion and replacement of the accessory genes with expression cassettes for puromycin-resistance and β-glucouronidase. Cell lines which produced in excess of 10^5 helper virus-free transducing units/ml were generated by *trans*-complementation of the replication defect using a BHK-21-derived cell line expressing the Bel-1 transactivator. Vectors based on the HFV genome may provide useful alternatives to existing retroviral vectors.

INTRODUCTION

The foamy viruses are a distinct group of retroviruses which, unlike lentiviruses and oncoviruses, have never been conclusively associated with natural infection (Ali *et al.,* 1996) or with any disease either in human or animals (Weiss 1993; Schweizer *et al.,* 1995). While the genome sequence of four isolates has been determined (Flugel *et al.,* 1987; Maurer *et al.,* 1988; Kupiec *et al.,* 1991; Renne *et al.,* 1992; Herchenröder *et al.,* 1994) and infectious molecular clones derived from human and chimpanzee isolates have been constructed (Rethwilm *et al.,* 1990; Löchelt *et al.,* 1991; Herchenroder *et al.,* 1995), this genera of viruses remains the least investigated of the Retroviridae.

Retroviral vectors have been exploited to study various aspects of the retrovirus life cycle, including reverse transcription and RNA packaging and are useful vehicles for gene transfer both *in vitro* and *in vivo* (reviewed by Miller, 1992). However, the unique replication strategy of foamy viruses (Rethwilm, 1996) has so far not allowed for the development of such packaging cells or vectors. In this study, replication-defective genomes derived from an infectious molecular clone of HFV (Rethwilm *et al.,* 1990) are described. Such retroviral vectors would serve as useful starting reagents for the mapping of minimal cis-acting sequences required for genome transfer. To this end, vector genomes were constructed which minimally disrupted the wild-type HFV sequence, while rendering genomes replication-defective. Furthermore, vector constructs were designed to minimize the probability that replication-competent viruses would be generated by recombination in vector producing cell lines.

Like other complex retroviruses (Cullen, 1992), HFV encodes a transcriptional transactivator (Bel-1) which is essential for LTR driven gene expression and for virus replication (Keller *et al.,* 1991; Rethwilm *et al.,* 1991; Löchelt *et al.,* 1991; Baunach *et al.,* 1993). In contrast, the *bel-2* and *bel-3* open reading frames are dispensible for HFV replication *in vitro* (Baunach *et al.,* 1993; Yu and Lineal, 1993). Therefore, a BHK-21-derived cell line (engineered to stably express Bel-1) was used in a similar manner to the adenovirus vector 293 helper cell line (Perricaudet and Perricaudet, 1995); i.e., to *trans*-complement defective HFV in which the *bel* genomic region was replaced with marker genes. Specifically, a vector provirus was constructed which replaced the *bel-1* and *bel-2* genes with a puromycin-resistance gene under the transcriptional control of the SV40 early promoter. A second vector, containing a *gusA* gene (encoding the bacterial enzyme β-glucuronidase) was similarly constructed. Bel-1 expressing cells were transfected with vector proviruses to generate cell lines producing virus particles capable of completing only the early stages of the retroviral life cycle, while expression of transduced genes could be monitored by antibiotic selection or histochemical staining.

MATERIALS AND METHODS

Molecular construction

A plasmid, pRFF62, which contains the HFV LTRs, *gag, pol,* and *env* genes with *bel-1* and *bel-2* reading frames destroyed (Fig. 1) was constructed. An SV40 promoter-

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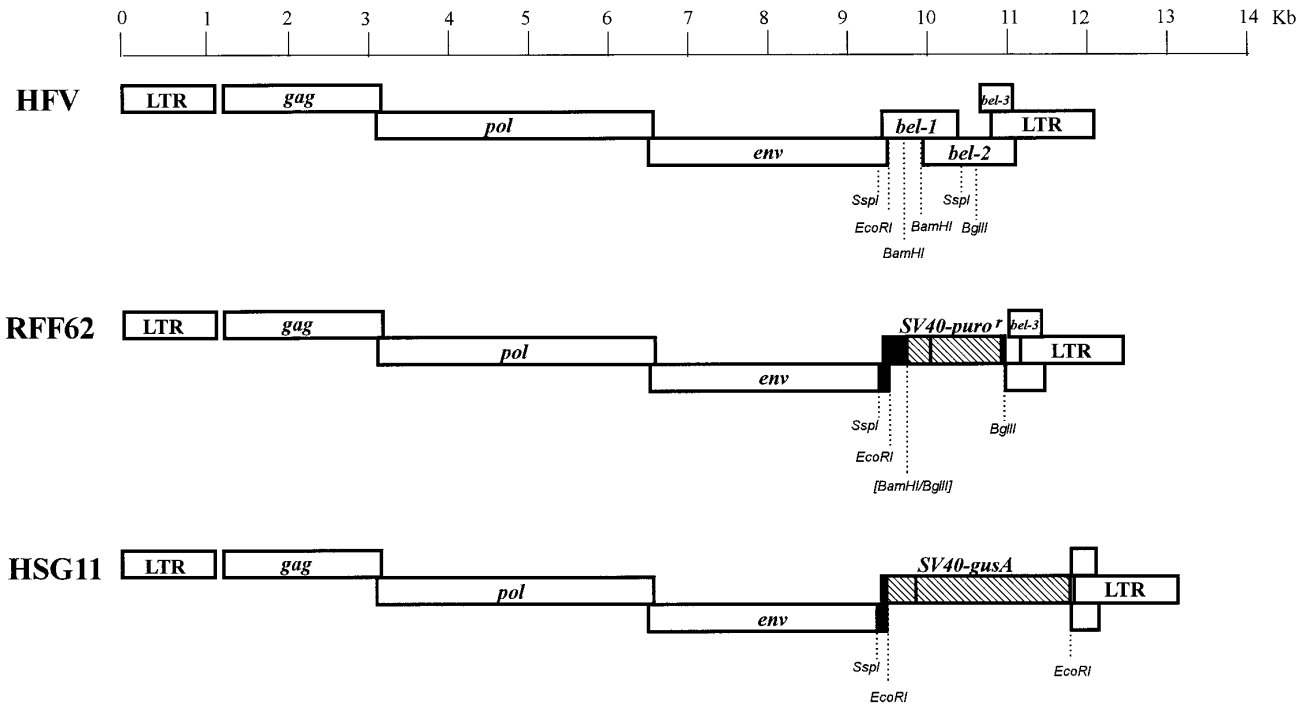


FIG. 1. HFV-derived vector constructs. Replication-defective vectors were constructed, as described in the text. The scale indicates nucleotide position in kilobases. Hatching indicates heterologous genes under the transcriptional control of an SV40 early promoter inserted into the *bel* genomic region. Solid shaded regions at the 3′ end of *env* and 5′ end of *bel-1* indicate sequences which are shared with the Bel-1 expression construct in BHK/Bel-1 cells.

puromycin-resistance gene (puro) cassette was first amplified from an MLV vector containing plasmid, pBabe*puro* (Morgenstern and Land, 1990) using polymerase chain reaction (PCR) primers which incorporated *Bgl*II sites (underlined) into each end of the amplicon (TACGTAGATCTTCGCCAGCACAGTGGT and TTACTAGATCTAGCTTGCCAAACCTAG). This PCR product was digested with *Bgl*II and inserted into *Bam*HI and *Bgl*II digested p3′komb, which contains sequences from the 3′ end of the *env* gene, *bel* genes, and the 3′ LTR (Rethwilm *et al.,* 1990). A clone with the insert in direct orientation relative to the viral coding sequences was identified. An *Nhe*I fragment consisting of the 3′ end of the *env* gene, the 5′ end of the *bel-1* gene, the SV40 *puro* cassette, and viral sequences 3′ of the *Bgl*II site was exchanged for the equivalent fragment of pHSRV1 which contains the infectious HFV genome (Rethwilm *et al.,* 1990). The final RFF62 vector genome is depicted in Fig. 1.

A second vector plasmid, pHSG11, containing the HFV LTRs, *gag, pol,* and *env* genes with *bel-1, bel-2,* and *bel-3* reading frames destroyed was constructed. A *Hin*dIII–*Nar*I fragment of pGUS358NS (Clontech) which includes the complete *Escherichia coli gusA* gene was inserted in place of the *puro* gene into *Hin*dIII- and *Cla*I-digested pBabe*puro* to create pBabe*gusA.* In addition, an *Eco*RI site was inserted into to the *Sma*I site of p3′komb-V1 [a modification of p3′komb with *bel-2* and *bel-3* sequences replaced by a poly-linker (Schmidt and Rethwilm, 1995)]. An *Eco*RI fragment of pBabe*gusA* consisting of the SV40 promoter and the *gusA* gene was inserted into the *Eco*RI sites of the modified p3′komb-V1 and a clone with the SV40 *gusA* cassette in direct orientation identified. This plasmid was digested with *Kpn*I and *Aat*II and a *Kpn*I–*Aat*II fragment of p5′komb (Rethwilm *et al.,* 1990) containing the 5′ LTR, *gag, pol,* and *env* sequences was inserted to reconstitute the complete vector genome, pHSG11 (Fig. 1).

Generation of BHK/Bel-1 cells

The *bel-1* gene containing 1.05-kb *Ssp*I fragment of pHSRV1 (Rethwilm *et al.,* 1990, 1991) was inserted into the *Eco*RI site of the retroviral vector pLEN (Adam and Miller, 1991) by blunt-end ligation. The resulting plasmid, pLEN*bel-1,* was transfected into the ecotropic packaging cell line GP+E-86 (Markowits *et al.,* 1988) and cells were selected in 1 mg/ml G418. BHK-21 cells were transduced with supernatant from the bulk GP+E-86/pLEN*bel-1* cells and selected with 0.5 mg/ml G418. Single cell clones were established from the G418-resistant cells by limiting dilution and screened by indirect immunofluorescence using a Bel-1 reactive rabbit antiserum, as previously described (Baunach *et al.,* 1993). The BHK/Bel-1 clone #28 was used for all further experiments and was characterized as follows (data not shown). Cell clone #28

showed exclusively nuclear staining with Bel-1 antiserum in immunofluorescence. In immunoblot a protein of 36 kDa apparent molecular weight which reacted with Bel-1 antiserum was detected in cellular lysates prepared from BHK/Bel-1#28, but not parental BHK-21 cells. Southern blot analysis revealed one copy of the bel-1 expression vector per cellular genome. Reporter gene assays using an HFV LTR*cat* construct (Erlwein and Rethwilm, 1993) demonstrated the functionality of the integrated *bel-1* gene.

Production of replication-defective viruses

BHK/Bel-1 cells were transfected using lipofectamine with 20 μg pRFF62 or 20 μg pHSG11 and 1 μg pPUR (Clontech). Forty-eight hours posttransfection cells were trypsinized and serially diluted in medium containing 2.5 μg/ml puromycin. Each dilution was distributed in the wells of 96-well plates (one complete plate per dilution). Selective medium was replaced every 3–4 days. After 10–14 days, cells were harvested from wells which contained single colonies. These colonies were individually expanded in 24-well plates (6 colonies in the case of RFF62 and 10 colonies in the case HSG11/pPUR), and each clone screened for viral antigen expression by immunoperoxidase staining with an HFV-positive human serum, as previously described (Bieniasz *et al.,* 1995a). In addition, pHSG11/pPUR transfectants were screened for β-glucuronidase expression by 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside/Nitroblue tetrazolium (X-gluc/NBT) staining, as described below. Clones which were 100% antigen-positive and, where appropriate, β-glucuronidase-positive were assayed for transfer of puromycin-resistance or β-glucuronidase expression to BHK-21 cells. To harvest vector stocks, producer cells were seeded in 150-cm^2 flasks and grown to confluence. The medium was then replaced with nonselective medium and the supernatant harvested 24 hr later, filtered (0.4 μm), aliquoted, and stored at $-70°$ or in liquid nitrogen.

Transduction of cells with HFV vectors

BHK-21 cells were seeded in 24-well plates at 2×10^4 cells/well the day before infection. Vector stocks were serially diluted 10-fold and 300 μl was inoculated into each well. The following day 500 μl of fresh medium was added. Forty-eight hours after inoculation, RFF62-transduced cells were trypsinized and transferred to 6-well plates containing growth medium and 2.5 μg/ml puromycin. Puromycin-resistant colonies were assayed after 10–14 days. HSG11 transduced cells were washed once with PBS and fixed for 10 min at room temperature with 1% formaldehyde/0.2% glutaraldehyde in PBS. After three washes with PBS, 0.4 μm filtered β-glucuronidase substrate solution (0.5 mg/ml X-gluc, 1 mg/ml NBT, 0.05% Triton X-100 in 100 m*M* Tris–HCl, pH 7.5) was added. Dark blue β-glucuronidase-positive cells were scored after overnight incubation at 37°.

Assays for helper virus

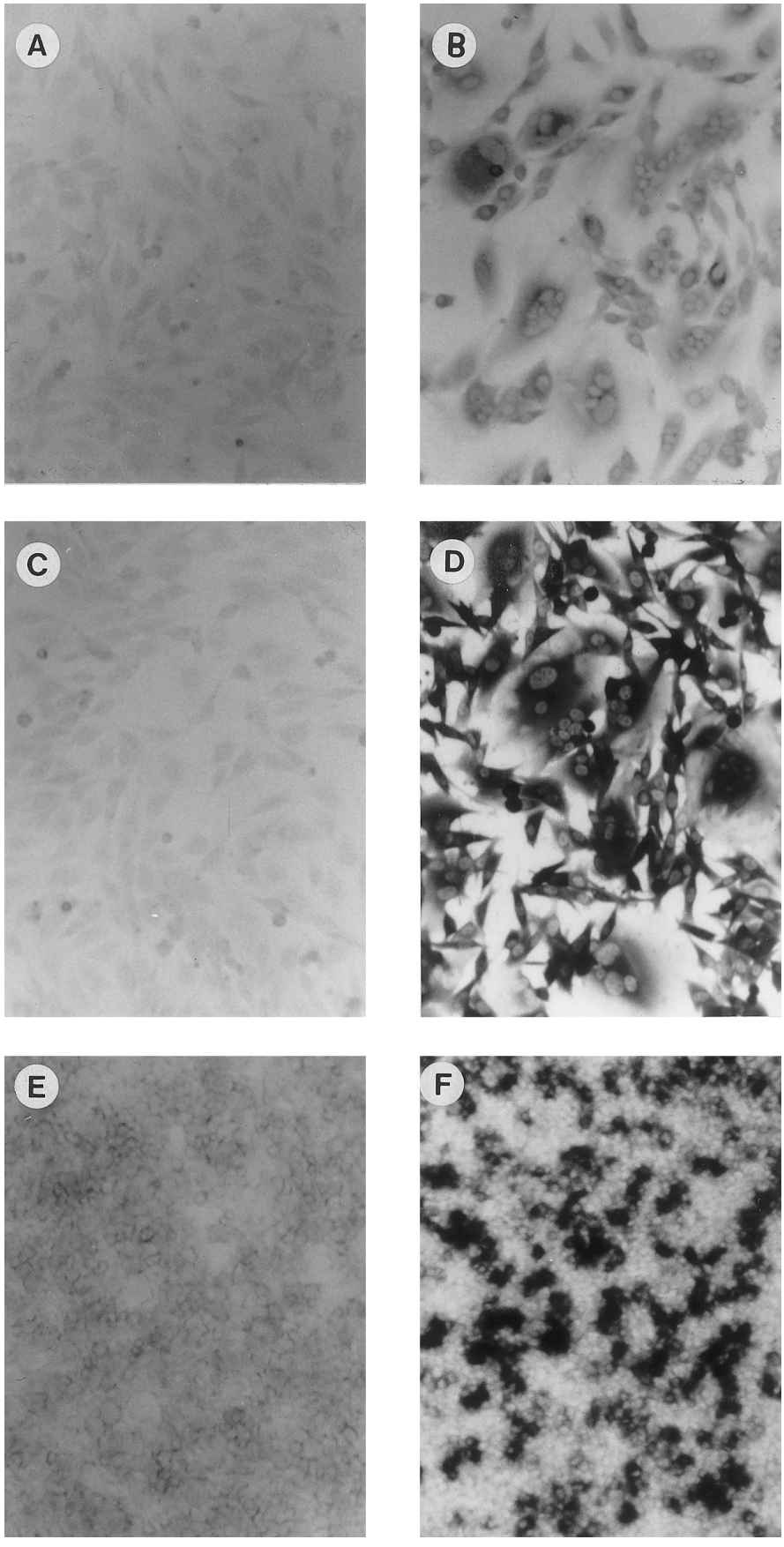
BHLL cells (Bieniasz *et al.,* 1995a) are BHK cells which contain an integrated *lacZ* gene under the transcriptional control of the HFV U3 promoter and, thus, form β-galactosidase-positive foci on infection with replication-competent HFV. To detect recombinant replication-competent helper-virus, BHLL cells were seeded at 10^5 cells/well in 6-well plates and on the following day each well was inoculated with a 1-ml aliquot of filtered supernatant harvested from RFF62 or HSG11 producing cells. Four hours later 2 ml fresh medium was added and the cells cultured for a further 48 hr. Cells were then fixed and stained for expression of β-galactosidase, as described previously (Bieniasz *et al.,* 1995a). Alternatively, 2×10^4 BHK-21 or BHK/Bel-1 cells were seeded in 24-well plates and the following day inoculated with 0.5-ml aliquots of 10-fold serially diluted vector supernatants. These cells were cultured for 48 hr and either immunoperoxidase stained using an HFV-positive human serum, or passaged in growth medium in the presence or absence of 2.5 μg/ml puromycin. After 1, 3, 5, and 7 passages supernatants were harvested and used to inoculate fresh BHK-21 cells. These cells were assayed for secondary vector transfer by selection in puromycin or by β-glucuronidase staining, as appropriate.

RESULTS

Stable cell lines producing replication-defective HFV vectors

Transient transfection of BHK/Bel-1 cells with either pRFF62 or pHSG11 resulted in HFV antigen expression, as detected by immunoperoxidase staining. However, this did not result in a spreading infection, as predicted. Over several passages the frequency of antigen-positive cells remained low, no cytopathic effect (CPE) was observed, and infection could not be reproducibly transmitted to fresh BHK/Bel-1 cells (data not shown). Presumably the bulk of BHK/Bel-1 cells which took up vector DNA during transient transfection did not produce sufficient virus to establish a spreading infection, i.e., the rate of proliferation of cells which did not express vector was greater than that of cells which did, and greater than their rate of infection. This may have been due to low level Bel-1 expression in BHK/Bel-1 cells. Puromycin selection and simultaneous cloning of transfected cells was carried out in order to isolate high titer producing cell clones, thereby preventing their loss due to outgrowth of cells in the culture which did not take up DNA during transfection.

Thus, directly following transfection, producer cell


A
B
C
D
E
F

lines were cloned in 96-well plates in the presence of 2.5 μg/ml puromycin. By this method, 6 RFF62-transfected clones and 10 HSG11/pPUR-transfected clones which remained viable on expansion in 24-well plates were isolated. After immunoperoxidase and X-gluc/NBT staining, 4 pRFF62 and 6 pHSG11/pPUR transfectants were identified which were 100% antigen-positive and, where appropriate, β-glucuronidase-positive (Figs. 2A–2D). Although the cell clones demonstrated varying levels of spontaneous cell fusion, they could be repeatedly passaged without obvious cell death. Thus, these clones were further analyzed for production of RFF62 and HSG11 virus vectors.

Transduction of *puro* and *gusA* genes

Initially, supernatant fluid from the four RFF62 producer clones and six HSG11 producer clones were screened for marker gene transduction. This was done by inoculation of BHK-21 cells in 24-well plates with 300 μl of undiluted, filtered supernatants, as described under Materials and Methods. On the basis of visual examination of puromycin-resistant colonies or X-gluc/NBT-stained cells, two RFF62 producer clones and four HSG11 producer clones which produced the highest vector titers were selected for further analysis. End-point titrations were carried out using assays designed to detect production of virions containing replication-defective vector genomes or recombinant replication-competent genomes (Table 1). Cell-free transduction of the *gusA* gene to fresh BHK-21 cells is shown in Figs. 2E and 2F. The titer of *puro*-transducing virus in the supernatant of the two RFF62 producer cell lines was $1-2 \times 10^5$ colony forming units/ml when assayed on BHK-21 cells (Table 1, column A). Similar titers of *gusA*-transducing virus ($5 \times 10^4-3 \times 10^5$) were detected in the supernatant of the four HSG11 producer clones (Table 1, column B). Successful transduction was achieved in different cell lines, for example, human embryonic lung (Hel), Hela, and a human rhabdomyosarcoma line (RD) (data not shown).

Helper virus detection and requirement for Bel-1 expression *in trans* for viral gene expression

RFF62 and HSG11 stocks were routinely assayed for the presence of helper virus by inoculation of BHLL cells. Alternatively, transduced BHK-21 cells were assayed for viral antigen expression by immunoperoxidase staining. In several cases, replication-competent virus was not detected by immunoperoxidase staining or BHLL assays (Table 1, columns E and F). However, two clones (RFF62T and HSG11/11N) produced low titers of replication-competent virus. In contrast, when BHK/Bel-1 cells were used as target cells for RFF62 or HSG11, viral antigen-positive cells could be detected by immunoperoxidase staining (Table 1, column D).

Although the HSG11 titers obtained using BHK/Bel-1 target cells were lower than those obtained using BHK-21 cells (Table 1, columns B and C), this was also the case for wild-type HFV (Table 1, columns D and E), indicating that this was an inherent property of the BHK/Bel-1 cell line, and was not a vector-specific phenomenon. Where examined, the titers were similar when BHK/Bel-1 cells were used as targets for the HSG11 vector, and either immunoperoxidase staining or X-gluc/NBT staining as a measure of infectivity (Table 1, column C and D).

Overall, these results indicate that the vector genomes were transferred in largely intact form, with rare generation of replication competent helper virus by recombination in BHK/Bel-1 cells.

Requirement for Bel-1 expression *in trans* for secondary vector transfer

While immunoperoxidase staining and BHLL assays are both sensitive and specific for replication-competent virus arising by recombination, negative results in these experiments do not exclude the possibility that low titers of vector might be produced by transduced Bel-1-negative BHK-21 cells (via basal transcription directed by a non-*trans*-activated LTR). Therefore, secondary transfer of puromycin-resistance or β-glucuronidase expression was attempted: BHK-21 cells were inoculated with 10^5 CFU of RFF62 or 10^5 FFU of HSG11 (from RFF62/2N and HSG11/22N producer clones, respectively) and after 1, 3, 5, and 7 passages either in the presence (for RFF62 transduced cells only) or absence of puromycin selection culture supernatants harvested for secondary transfer to BHK-21 cells. In neither case was secondary transfer of vector genomes demonstrable (vector titer of <1). In contrast, when Bel-1 was provided *in trans* (by inoculation of BHK/Bel-1 cells with RFF62 or HSG11 vectors) secondary transfer of *puro* and *gusA* was readily demonstrated. Although this secondary transfer was inefficient with unselected, vector inoculated BHK/Bel-1 cells (producing vector titers of 60 and 120 after inoculation with RFF62 and HSG11, respectively), selection of RFF62-transduced BHK/Bel-1 cells (pooled puromycin-resistant colonies) resulted in secondary transfer titers in excess of 10^4 CFU/ml.

FIG. 2. HFV vector producing cell lines and Transduction of BHK-21 with the gusA gene using the HSG11 vector. Immunoperoxidase staining of BHK/Bel-1 cells (A) and the RFF62/2N producer clone (B) using an HFV-positive human serum. X-gluc/NBT staining of BHK/Bel-1 cells (C) and the HSG11/9S producer clone (D); BHK-21 cells (2×10^4/well in 24-well plates) were mock inoculated (E) or inoculated with 100 μl filtered supernatant from the HSG11/22N clone (F) and assayed for *gusA* transduction by X-gluc/NBT staining 48 hr postinoculation.

TABLE 1

Transfer of *puro* and *gusA* Genes and Assays for Helper Virus

Virus/vector titer in supernatants taken from producer cell clones (measured using BHK-21, BHK/Bel-1, or BHLL target cells)						
Virus/vector virus/vector producer line	A Puromycin-resistant CFU/ml (BHK-21)	B β-glucuronidase$^+$ FFU/ml (BHK-21)	C β-glucuronidase$^+$ FFU/ml (BHK/Bel-1)	D Immunoperoxidase$^+$ FFU/ml (BHK/Bel-1)	E Immunoperoxidase$^+$ FFU/ml (BHK-21)	F β-galactosidase$^+$ FFU/ml (BHLL)
RFF62/2N	2×10^5			6×10^3	<1	<1
RFF62/T	1×10^5			ND[a]	1	2
HSG11/22N		3×10^5	2×10^4	1.5×10^4	<1	<1
HSG11/11N		7×10^4	6×10^3	9×10^3	20	28
HSG11/3S		5×10^4	ND	ND	<1	<1
HSG11/9S		1×10^5	3×10^3	5×10^3	<1	<1
BHK/HFV				5×10^4	2.5×10^5	3×10^5

Note. Assays were chosen to detect transfer of replication-defective vector genomes (columns A, B, and C), replication-competent virus (columns E and F), or both (columns D). Column A indicates the puromycin-resistant colony-forming titer using BHK-21 target cells in supernatants harvested from RFF62 producing clones. Columns B and C indicate the β-glucuronidase-positive focus-forming titer (using BHK-21 and BHK/Bel-1 target cells, respectively) in supernatants harvested from HSG11 producing clones. Columns D and E indicate the viral antigen-positive focus-forming titer (using BHK/Bel-1 and BHK-21 target cells, respectively) in vector supernatants. Column F indicates the β-galactosidase-positive focus-forming titer using BHLL target cells.

[a] ND, not done.

Stability of vector genome expression

The results described above and in Table 1 were obtained using supernatants harvested from producer cells within 2 months (approximately 15 passages) of their initial cloning. To assess the stability of vector expression, one producer clone (HSG11/22N) which was initially helper virus-negative, was passaged for a further 3 months (approximately 40 passages in total). By this time, spontaneous cell fusion had diminished (as compared to the freshly isolated clone). Supernatant was then harvested and vector titer measured by X-gluc/NBT staining and helper virus tests were performed using BHLL cells. The *gusA* transducing titer had declined to 7×10^4 FFU/ml, and no helper virus was detected in 2.5 ml of supernatant.

DISCUSSION

In this study, cell lines producing replication-defective HFV-based vectors were constructed. In addition to their potential use in receptor identification, these vectors should be useful starting reagents in experiments designed to map *cis*-acting packaging sequences in the HFV genome, an essential step in the development of HFV as a potential vector for therapeutic gene transfer. From this study, it is evident that *cis*-acting sequences required for vector expression or packaging are not located in the *bel*-genomic region.

Although most cell lines analyzed produced only-replication-defective virus, some vector producing cell lines also produced low titers of virus which resulted in antigen-positive foci or β-galactosidase-positive foci following inoculation of BHK-21 and BHLL indicator cells, respectively. This replication-competent virus presumably arose by recombination between the vector genomes and the Bel-1 expression construct. Since the HFV env and *bel-1* genes overlap (Flügel *et al.,* 1987) there were homologous sequences present in both constructs at the 5′ end of the *bel-1* gene. The stretches of sequence homology between the integrated *bel-1* gene and the vectors RFF62 and HSG11 are 540 and 145 nt in length, respectively (Fig. 1). However, only RFF62 contains MLV-derived sequences (originally from pBabe*puro*) at the 3′ end of the inserted reporter gene. Therefore, nonhomologous recombination between vector and Bel-1 expression construct would be required to reconstitute replication-competent virus in HSG 11 producing cells. Whether this recombination occurred between DNA molecules in the producer cells, or by interstrand transfer during reverse transcription is unknown.

Two previous studies have exploited vectors based on the HFV genome for the transfer of marker genes *in vitro.* Vectors have been constructed in which sequences between the 3′ end of the *bel-1* gene and the 3′ LTR were replaced with a variety of marker genes (Schmidt and Rethwilm, 1995). Since *gag, pol, env,* and *bel-1* genes remained intact, these vectors remained replication-competent and were produced in similar titers to wild-type HFV. Marker genes were encoded by RNAs that normally express the viral Bet protein either as intact proteins or as Bet-fusion proteins.

Complementary to these studies, replication-defective vectors have been produced by insertion of marker genes in place of the HFV *env* gene (Russell and Miller, 1996).

Vectors were packaged by transient cotransfection with an HFV *gag/pol* deletion mutant or wild-type virus. Even when the defective *trans*-complementing virus was used, replication-competent helper virus was generated by recombination and transfer of marker genes was accompanied by similar titers of replication-competent HFV.

Foamy virus-based vectors may have several advantages over existing retroviral vectors for gene for *in vivo* gene transfer: (i) The large packaging limit of foamy viruses (Schmidt and Rethwilm, 1995) means that larger genes could be transferred than is currently possible with existing retroviral vectors, provided that elements which are sufficient to direct genome encapsidation can be identified. (ii) The recent finding that Pol proteins are expressed independently of Gag (Enssle *et al.,* 1996; Lochelt and Flugel, 1996; Yu *et al.,* 1996) and that virus mutants functionally deficient in either Gag or Pol expression can complement each other *in trans* means that, in theory, packaging cell lines could be constructed where Gag, Pol, and Env are synthesized from three separate expression constructs. This would reduce the possibility of generating replication-competent helper virus by recombination in packaging cells. (iii) Since the HFV promoters are not competent to support virus replication in the absence of Bel-1, vector constructs which essentially eliminate the possibility of generating replication-competent virus could be designed: U3 sequences in the 5′ LTR would be replaced by a constitutive promoter, eliminating the requirement for Bel-1 for vector RNA synthesis. Such a vector packaged in a cell line engineered to express Gag, Pol, and Env *in trans* under the control of heterologous promoters would constitute a safe gene transfer mechanism, since an essential gene for virus replication is completely excluded from the system. (iv) In common with vectors based on oncoviruses, a major limitation of the use of foamy virus-based vectors is the requirement for cell proliferation in optimal infection (Bieniasz *et al.,* 1995; Russell and Miller, 1996), despite the presence of a peptide signal in HFV nucleocapsid protein which directs nuclear transport of HFV Gag precursor late in the viral life cycle; i.e., after genome integration and expression (Shliephake and Rethwilm, 1994). However, if efficient nuclear targeting of preintegration complexes can be achieved, then foamy viruses may be less restricted in infection of quiescent cells containing low levels of nucleosides, since the bulk of reverse transcription may occur in virus producing cells (Yu *et al.,* 1996).

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